Aromatic metabolites from the coelomic fluid of Eisenia earthworm species

Rochfort, Simone; Wyatt, Morgan A.; Liebeke, Manuel; Southam, Andrew D.; Viant, Mark R.; Bundy, Jacob G.

DOI:
10.1016/j.ejsobi.2016.11.008

European Journal of Soil Biology 78 (2017) 17–19

Contents lists available at ScienceDirect

European Journal of Soil Biology

journal homepage: http://www.elsevier.com/locate/ejsobi

Aromatic metabolites from the coelomic fluid of *Eisenia* earthworm species

Simone Rochfort [a, b], Morgan A. Wyatt [c], Manuel Liebeke [c, 1], Andrew D. Southam [d], Mark R. Viant [d], Jacob G. Bundy [c, *]

[a] Biosciences Research, Department of Economic Development, Jobs, Transport and Resources, AgriBio, 5 Ring Road, Bundoora, Victoria, 3083, Australia
[b] School of Applied Systems Biology, La Trobe University, Bundoora, Victoria, 3083, Australia
[c] Imperial College London, Department of Surgery and Cancer, Sir Alexander Fleming Building, London, SW7 2AZ, UK
[d] School of Biosciences, University of Birmingham, Birmingham, B15 2TT, UK

ARTICLE INFO

Article history:
Received 22 July 2016
Received in revised form
25 November 2016
Accepted 28 November 2016
Available online 8 December 2016

Handling Editor: S. Schrader

Keywords:
Earthworms
Coelomic fluid
Metabolites
Riboflavin

ABSTRACT

Earthworms from the genus *Eisenia* express coelomic fluid when under severe stress. This coelomic fluid contains a complex mixture of small-molecule metabolites, including aromatic metabolites which are known to be species-specific, yet their actual identities remain unknown. We have aimed to characterize selected high-concentration coelomic fluid metabolites. The major aromatic compound in *Eisenia veneta* coelomic fluid is the rare metabolite α-nicotinamide riboside; and the major aromatic compound for *Eisenia fetida* is closely related to the (already characterized) metabolite of *Eisenia andrei*, which consists of two aromatic quinazoline-2,4-dione ring structures linked by *N*-acetylspermine. The biological function(s) of these metabolites in earthworms is unknown, but we hypothesize that they represent remnants of larger molecules, possibly bacterial in origin, that are recalcitrant to metabolism by earthworm enzymes.

The closely-related species *Eisenia fetida* and *E. andrei* can be distinguished by colour [1], but are not otherwise separated by standard keys [2]. They were widely accepted as separate species only following the demonstration of electrophoretic separation of different protein isoforms between them [3,4], and they do not produce fertile crosses [5]. The precise taxonomic status of these species is complex and may require revision [6], but that is not our current concern. Instead, we are interested in the functional differences that underlie the separation of these closely-related species at the molecular level. Both species exude a pungent yellow liquid on rough handling or stress, which we refer to as exuded coelomic fluid (ECF). The yellow colour comes from riboflavin, which is found partly in the fluid, and partly associated with the large number of coelomocytes that are also released; Albani et al. [7] reported riboflavin in *E. andrei*, and Koziol et al. [8] also observed it in *E. fetida*. However, ECF also contains a complex mixture of small-molecule metabolites; the *Eisenia veneta* ECF is dominated by organic acids, including the citric acid cycle intermediates fumarate, succinate, malate, and α-ketoglutarate [9]. The *E. fetida* ECF also contains organic bases such as spermidine and putrescine, and membrane components such as *myo*- and *scyllo*-inositol [10]. Earthworm coelomic fluid has long been of interest as a complex matrix with many biological functions, including immune defence [11]; but in the context of excretion and transport of small molecules, most interest has been in the excretory system of the nephridiopore, with little attention paid to the coelomic fluid metabolite composition [12]. The untargeted NMR analyses show that the coelomic fluid has a high metabolic complexity.

An earlier study reported that, although NMR-based metabolic profiling of tissue extracts showed essentially no differences between *E. fetida* and *E. andrei*, their ECF profiles were dramatically different, particularly in signals from unidentified aromatic metabolites [13]. The species *E. veneta* had further differences in the aromatic metabolites present. However, these metabolites were not identified. Here, we assign the principal aromatic compound from *E. veneta*, and provide further evidence regarding the principal aromatic compound from *E. fetida*, and show that they are uncommon and unexpected metabolites, with unknown function in

* Corresponding author.
E-mail address: j.bundy@imperial.ac.uk (J.G. Bundy).
[1] Current address: Department of Symbiosis, Max-Planck-Institute for Marine Microbiology, Bremen, 28359, Germany.

http://dx.doi.org/10.1016/j.ejsobi.2016.11.008

earthworms.

We extracted a large amount of ECF by treating many *E. fetida* and *E. andrei* (approximately 2000 per species) with 10% NaCl solution (1 L), which causes rapid expulsion of ECF. The worms were obtained from Blades Biological (Edenbridge, UK), and were gently washed in tap water before extraction to remove the residual compost medium. The coelomocytes are also generally lysed by this treatment, but we added an additional extraction step of the cell and debris pellet (following centrifugation) with ~4 vol of methanol. We then used a C18 solid-phase extraction (SPE) cartridge (Sigma-Aldrich, UK) to desalt and concentrate the aromatic metabolites, before fractionating the sample using HPLC, using a 10 × 250 mm ACE C18-AQ column (Hichrom, UK), eluting with a gradient of 0.1% aqueous ammonia solution to 30% acetonitrile, with UV detection at A_{254}. Individual chromatographic peaks were collected manually in glass vials and dried down before resuspending in solvents for NMR and/or MS analysis. High-resolution FT-ICR mass spectrometry was performed using an LTQ FT Ultra (Thermo Fisher Scientific, Bremen, Germany) equipped with a chip-based direct infusion nanoelectrospray ion source (Triversa, Advion Biosciences, Ithaca, NY) in positive mode.

We also treated a much smaller number of *E. veneta*, about 100, in the same way to obtain a series of purified metabolites.

For *E. veneta* (obtained from a culture maintained at CEH Wallingford), we obtained higher quality 2D NMR spectra than we had managed before. Based on the comparison of the spectra to data reported in the literature [14], we assigned the main *E. veneta*-specific aromatic metabolite as α-nicotinamide riboside; this had previously been incorrectly described as nicotinamide mononucleotide [9], i.e. the actual metabolite is the nucleoside and not the nucleotide, and the α rather than the more common β anomer.

For *E. andrei*, we collected an intensely coloured peak, which we confirmed to be riboflavin by NMR and MS comparison to an authentic standard. However, the largest peak belonged to a metabolite eluting at 26.5 min. *E. andrei* ECF contains an aromatic metabolite that has been previously referred to only as SP-8203; it consists of two quinazoline-2,4-diones joined by an N-acetylspermine linker [15]. However, the aromatic metabolite that is specific to *E. andrei* has 16 aromatic resonances [13], which would initially seem not to be compatible with SP-8203. We found that redissolving this compound in either water or in an aprotic solvent, DMSO-d_6, gave entirely different spectra. Varying the proportions of water and DMSO led to a smooth shift in the pattern of the aromatic peaks observed between the two extremes (Fig. 1A), which we ascribe to differences in the proportion of molecules being held in different conformations between the two solvents, with four stable and equimolar conformers in water, and two conformers, in roughly a 2:1 ratio, in DMSO.

We then acquired homo- and heteronuclear 2D NMR data, using the (simpler) DMSO solution, and conclude that the previously unassigned metabolite is indeed SP-8203. (The ^{1}H and ^{13}C shifts for this metabolite have not previously been reported in the open literature, and are given in online supplementary information.) Furthermore, a high-resolution mass spectrum of this fraction had a major peak with 535.2666 *m/z*, which compares well to the predicted formula of 535.2669 for the $[M+H]^+$ ion.

The *E. fetida*-specific metabolite gave rise to a peak in the mass spectrum at 510.2709 *m/z*, which corresponds to a possible molecular formula (for $[M+H]^+$) of $C_{27}H_{36}N_5O_5$ (calculated mass 510.2711, mass error = 0.43 ppm), representing a difference of +H and −C, −N, from SP-8203. This immediately suggests a possible related metabolite structure (Fig. 1B), although the actual structure remains to be confirmed unambiguously.

Why do *Eisenia* species contain these unusual aromatic metabolites? It has been suggested that some of these metabolites may

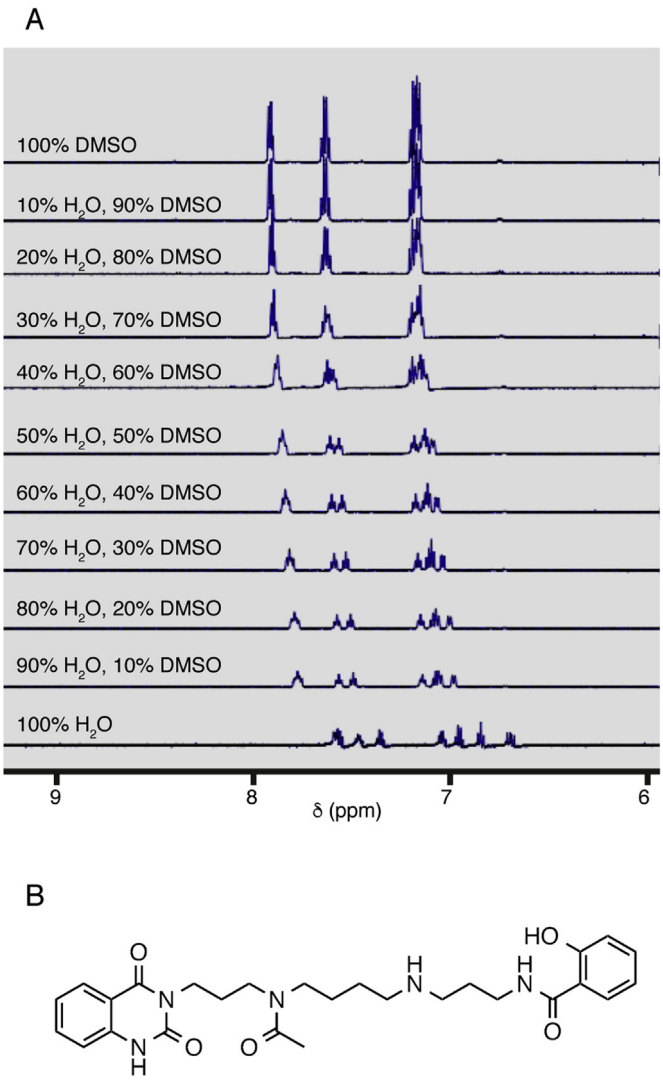

Fig. 1. A: Increasing the proportion of water to DMSO in the sample changes the apparent number of aromatic peaks of the *Eisenia andrei* metabolite SP-8203. The spectra represent mixtures of 100% DMSO (top) to 100% water (bottom), in 10% steps. Aromatic region only is shown. B: A possible structure for the major aromatic metabolite from *Eisenia fetida* coelomic fluid.

have direct functional roles – for instance, riboflavin is found in a wide number of earthworm species, and may play a direct role in earthworm immunity [16]. The compound SP-8203 is highly pharmacologically potent towards a number of endpoints in mammalian cells [17,18], although as yet nothing is known about its activity in its native earthworm host. An alternative hypothesis is that these metabolites may be accumulated breakdown products. Animals cannot synthesize riboflavin, and so all the riboflavin in ECF must be microbial in origin [19]. Earthworm coelomocytes include phagocytic immune cells [20], and potentially riboflavin could accumulate in coelomocytes and coelomic fluid from phagocytosed bacteria. Free α-nicotinamide riboside has only been reported once previously to our knowledge, as an antifouling metabolite from a sponge [21]. However, α-NADH can probably be epimerized *in vivo* from β-NADH, and then enzymatically converted to α-NAD [22]. It is plausible that the earthworm enzymes can readily degrade microbial β-NAD (the normal form), but that when α-NAD is present, they can metabolize the adenosine nucleotide part, but α-nicotinamide riboside is left behind as a recalcitrant

residue. Earthworms adapt their 'normal' biochemical enzymes for phase II metabolism of xenobiotics [23], which could also potentially explain the quinazoline-dione metabolites, SP-8203 (from *E. andrei*), and the related compound from *E. fetida* ($C_{27}H_{35}N_5O_6$). *Lumbricus terrestris* has been previously reported to conjugate the pesticide cypermethrin with spermine [24], and it is easy to see how SP-8203 could be derived from spermine (or *N*-acetylspermine) conjugation of smaller units. Again, therefore, SP-8203 and the *E. fetida* metabolite could be produced from other endogenous metabolites, and may not represent functional compounds in earthworms. We acknowledge that it is still, of course, possible that these metabolites may be directly functional in worms; similar to the proposed role of riboflavin in immunity, for instance [16]. Future work is needed both to characterize the responses of these metabolites to external factors, such as infection or stress, as well as to test their function directly.

There remains to be explained the striking differences between *Eisenia* species – why is α-nicotinamide riboside found exclusively in *E. veneta*, and different unique quinazoline-dione metabolites found exclusively in *E. andrei* and *E. fetida*? We do not have an answer, beyond noting here that the differences may lie either in the earthworms' own enzymatic capabilities, or in the associated microbiomes of the different species. This raises interesting questions for biologists interested in evolution of functional differences between closely-related species [25]. Furthermore, a better understanding of the possible source of metabolites in ECF will benefit future studies of changes in these metabolites in response to toxic or other environmental stress [10,26].

Acknowledgements

This study was supported by the Natural Environment Research Council (NERC), UK, under grant number NE/H009973/1. JGB also thanks the Department of Primary Industries, Victoria, Australia, for support as an Overseas Research Lecturer. We thank David Spurgeon and Claus Svendsen (CEH Wallingford, UK) for supplying *Eisenia veneta* worms.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejsobi.2016.11.008.

References

[1] F. André, Contribution a l'Etude Expérimentale de la Reproduction des Lombriciens, Bull. Biol. Fr. Belg. 97 (1963) 4–101.
[2] R.W. Sims, B.M. Gerard, Earthworms: Keys and Notes for Identification of the Species, Linnean Society, London, 1985.
[3] J. Jaenike, "*Eisenia foetida*" is two biological species, Megadrilogica 4 (1982) 6–8.
[4] N. Øien, J. Stenersen, Esterases of earthworms—III. Electrophoresis reveals that *Eisenia fetida* (Savigny) is two species, Comp. Biochem. Physiol. Part C Comp. Pharmacol. 78 (1984) 277–282, http://dx.doi.org/10.1016/0742-8413(84)90083-5.
[5] J. Domínguez, A. Velando, A. Ferreiro, Are *Eisenia fetida* (Savigny, 1826) and *Eisenia andrei* (Oligochaeta, Lumbricidae) different biological species? Pedobiologia 49 (2005) 81–87, http://dx.doi.org/10.1016/j.pedobi.2004.08.005.
[6] M.J.I. Briones, P. Morán, D. Posada, Are the sexual, somatic and genetic characters enough to solve nomenclatural problems in lumbricid taxonomy? Soil Biol. Biochem. 41 (2009) 2257–2271, http://dx.doi.org/10.1016/j.soilbio.2009.07.008.
[7] J.R. Albani, S. Demuynck, F. Grumiaux, A. Leprêtre, Fluorescence fingerprints of *Eisenia fetida* and *Eisenia andrei*, Photochem. Photobiol. 78 (2003) 599–602, http://dx.doi.org/10.1562/0031-8655(2003)0780599FFOEFA2.0.CO2.
[8] B. Koziol, M. Markowicz, J. Kruk, B. Plytycz, Riboflavin as a source of autofluorescence in *Eisenia fetida* coelomocytes, Photochem. Photobiol. 82 (2006) 570–573, http://dx.doi.org/10.1562/2005-11-23-RA-738.
[9] J.G. Bundy, D. Osborn, J.M. Weeks, J.C. Lindon, J.K. Nicholson, An NMR-based metabonomic approach to the investigation of coelomic fluid biochemistry in earthworms under toxic stress, FEBS Lett. 500 (2001) 31–35.
[10] J. Yuk, M.J. Simpson, A.J. Simpson, Coelomic fluid: a complimentary biological medium to assess sub-lethal endosulfan exposure using ^{1}H NMR-based earthworm metabolomics, Ecotoxicology 21 (2012) 1301–1313, http://dx.doi.org/10.1007/s10646-012-0884-5.
[11] M. Lassegues, P. Roch, P. Valembois, Antibacterial activity of *Eisenia fetida andrei* coelomic fluid - evidence, induction, and animal protection, J. Invertebr. Pathol. 53 (1989) 1–6, http://dx.doi.org/10.1016/0022-2011(89)90066-9.
[12] L.B. Kirschner, Comparative physiology: invertebrate excretory organs, Annu. Rev. Physiol. 29 (1967) 169–196, http://dx.doi.org/10.1146/annurev.ph.29.030167.001125.
[13] J.G. Bundy, D.J. Spurgeon, C. Svendsen, P.K. Hankard, D. Osborn, J.C. Lindon, J.K. Nicholson, Earthworm species of the genus *Eisenia* can be phenotypically differentiated by metabolic profiling, FEBS Lett. 521 (2002) 115–120, http://dx.doi.org/10.1016/S0014-5793(02)02854-5.
[14] N.J. Oppenheimer, N.O. Kaplan, Proton magnetic resonance study of the intramolecular association and conformation of the alpha and beta pyridine mononucleotides and nucleosides, Biochemistry 15 (1976) 3981–3989.
[15] Y.H. Choi, J.H. Suh, J.H. Lee, I.H. Cho, C.K. Lee, M.G. Lee, LC–UV analysis of N-{3-(2,4-Dioxo-1,4-dihydro-2H-quinazolin-3-yl)propyl}-N-[4-{3-(2,4-dioxo-1,4-dihydro-2H-quinazolin-3-yl)propyl-amino}butyl]acetamide (SP-8203) in rat plasma, urine, and gastrointestinal tract samples, Chroma*tographia* 70 (2009) 1435–1439, http://dx.doi.org/10.1365/s10337-009-1327-1.
[16] A.I. Mazur, M. Klimek, A.J. Morgan, B. Plytycz, Riboflavin storage in earthworm chloragocytes and chloragocyte-derived eleocytes and its putative role as chemoattractant for immunocompetent cells, Pedobiologia 54 (2011) S37–S42, http://dx.doi.org/10.1016/j.pedobi.2011.09.008.
[17] S.-J. Noh, S.H. Lee, K.Y. Shin, C.K. Lee, I.H. Cho, SP-8203 reduces oxidative stress via SOD activity and behavioral deficit in cerebral ischemia, Pharmacol. Biochem. Behav. 98 (2011) 150–154, http://dx.doi.org/10.1016/j.pbb.2010.12.014.
[18] S.-J. Noh, J.M. Lee, K.S. Lee, H.S. Hong, C.K. Lee, I.H. Cho, H.-S. Kim, Y.-H. Suh, SP-8203 shows neuroprotective effects and improves cognitive impairment in ischemic brain injury through NMDA receptor, Pharmacol. Biochem. Behav. 100 (2011) 73–80, http://dx.doi.org/10.1016/j.pbb.2011.07.018.
[19] I. Haase, T. Gräwert, B. Illarionov, A. Bacher, M. Fischer, Recent advances in riboflavin biosynthesis, Methods Mol. Biol. 1146 (2014) 15–40, http://dx.doi.org/10.1007/978-1-4939-0452-5_2.
[20] E.L. Cooper, E. Kauschke, A. Cossarizza, Digging for innate immunity since Darwin and Metchnikoff, Bioessays 24 (2002) 319–333, http://dx.doi.org/10.1002/bies.10077.
[21] T. Hattori, S. Matsuo, K. Adachi, Y. Shizuri, Isolation of antifouling substances from the Palauan sponge *Protophlitaspongia aga*, Fish. Sci. 67 (2001) 690–693, http://dx.doi.org/10.1046/j.1444-2906.2001.00307.x.
[22] N.J. Oppenheimer, N.O. Kaplan, The alpha beta epimerization of reduced nicotinamide adenine dinucleotide, Arch. Biochem. Biophys. 166 (1975) 526–535.
[23] B.D. Sloley, gamma-Glutamyl conjugation of 5-hydroxytryptamine (serotonin) in the earthworm (*Lumbricus terrestris*), Neurochem. Res. 19 (1994) 217–222.
[24] E.A. Curl, P.J. Edwards, C. Elliott, J.P. LeAhey, The conjugation and accumulation of metabolites of cypermethrin by earthworms, Pestic. Sci. 20 (1987) 207–222, http://dx.doi.org/10.1002/ps.2780200306.
[25] T.C. McElroy, W.J. Diehl, Heterosis in two closely related species of earthworm (*Eisenia fetida* and *E. andrei*), Hered. (Edinb) 87 (2001) 598–608.
[26] J. Yuk, M.J. Simpson, A.J. Simpson, 1-D and 2-D NMR-based metabolomics of earthworms exposed to endosulfan and endosulfan sulfate in soil, Environ. Pollut. 175 (2013) 35–44, http://dx.doi.org/10.1016/j.envpol.2012.12.007.